EXHIBIT D

IMMUNOHISTOCHEMISTRY PROTOCOLS

The Antibody

We designed a fusion protein containing an area close to the putative pore region (residues 374 to 452 of SEQ ID NO: 3, EAG1.62.mAb) and one in the carboxy terminus of EAG (residues 872 to 932 of SEQ ID NO: 3). Hybridomas were generated by BioGenes GmbH (Berlin, Germany) using standard techniques. The monoclonal antibodies were screened for their ability to bind the fusion protein as compared to the closely related protein known (EAG2) using surface plasmon resonance (Biacore, Uppsala, Sweden). Those supernatants with the best performance in this test were then examined by immunohistochemistry on human brain tissues. The hybridomas giving the expected staining patterns were subcloned.

The antibodies used in this study were subsequently purified by affinity chromatography, first on a protein A column and then on a second affinity column with the fusion protein used for immunization. The activity of the antibody was again checked by surface plasmon resonance prior to use.

We assessed the specificity of EAG1.62.mAb for EAG protein. We assessed the ability of EAG1.62.mAb to recognize EAG1 in Chinese hamster ovary (CHO) cells transiently transfected with *EAG*. We cloned the EAG nucleotide sequence into vector pTracerCMV, therefore, EAG was expressed with the green fluorescent protein (GFP), which was visible in transfected cells. In these experiments, immunohistochemical localization of EAG1 was detected using EAG1.62.mAb and a red-fluorescent secondary antibody. We observed that red fluorescence was detectable only in the cells that emitted green fluorescence

due to GFP. This indicated that the EAG1.62.mAb antibody labeled only those cells expressing EAG1.

We also performed transient transfections with a chimeric fusion protein containing the entire EAG1 channel protein with the enhanced GFP attached to its amino-terminus (EGFP–hEAG1). The characterization of the chimera showed that the properties of EAG1 are preserved (not shown). These cells were immunostained with EAG1.62.mAb and a red fluorescent secondary antibody. We observed that the red fluorescence due to antibody binding precisely colocalized with the green fluorescence marking the channel. Thus, the antibody bound only to those cells that had been transfected with EAG1 and only to the areas where the channel was localized. Indistinguishable staining patterns were obtained when using the monoclonal antibody against a different epitope in the carboxyl terminus of EAG1 (EAG1.33.mAb, data not shown).

The two monoclonal antibodies, EAG1.62.mAb and EAG1.33.mAb showed cross-reactivity to the rat EAG. We obtained virtually identical immunohistochemical staining patterns with both antibodies EAG1.62.mAb and EAG1.33mAb that in both cases overlapped with the pattern described for rat EAG1. It should be understood that one of ordinary skill in the art could easily develop anti-EAG antibodies that specifically recognize the human EAG proteins of this application using standard techniques. For example, one of ordinary skill in the art would know how to screen monoclonal antibodies that bind the human EAG protein, but not human EAG2 by ELISA assay

Immunohistochemistry

Tissue array slides were dried for 1 hour in an oven at 58°C. Tissue samples were deparafinned by immersion in xylene for 5 x 4 minutes. The slides were subsequently hydrated by graded ethanol immersions (100%, 95%, 70%) for 2 x 3 minutes per ethanol. Finally, slides were immersed in tap water for 5 minutes.

Antigen retrieval was performed in a microwave oven in 10 mM citrate buffer (pH 6.0) at 700 W for at least 15 minutes before being immersed in cold Tris-buffered saline (TBS).

Endogenous peroxidase activity was quenched by immersing slides in 0.03% hydrogen peroxide solution containing 0.2% NaN_3 for 5 minutes, followed by rinsing in water and 3 x 5 minutes washes in TBS. Slides were then pre-blocked with serum (1:50 dilution) for 20 minutes followed by incubation with EAG1.62mAb overnight at 4°C. The slides were then washed 3 x 5 minutes in TBS and incubated with peroxidase-labeled polymer (DAKO Diagnostica, Hamburg, Germany) for 30 minutes, washed and incubated with diaminobenzidine (DAB) solution for 5 minutes. The chromogenic reaction was stopped by rinsing in tap water. The slides were counterstained in Haematoxylin (nuclear stain) for 10 seconds and dehydrated in graded ethanol (75%, 80%, 85% and 100%). Finally, the slides were cleared in xylene 4 x 5 minutes and mounted with Permount.

Analysis

Cells testing visually positive were counted. Antigen expression, that is, EAG protein expression, was presented as an immunoreactive score (IRS). The signal intensity (SI) of the overall slide was given a value from 0 (negative) to 3 (intensive). The staining amount (SA) value was determined by counting the number of cells stained out of the total cells and assigning a value for the percentage of stained cells as follows: 1, 0%–10% stained

cells; 2, 11%–50% stained cells; and 3, >50% stained cells. The maximum IRS was calculated by adding the highest SI and SA values for each sample. The baseline IRS was taken as the IRS calculated from fibroblasts in the same sample. Tumor-free tissue served as negative control, and mast cells (staining intensity (SI) = 3) served as positive controls.